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PPAR γ agonists do not directly enhance basal or insulin-stimulated Na⁺ transport via the epithelial Na⁺ channel

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Abstract Selective agonists of peroxisome proliferator-activated receptor gamma (PPAR γ) are anti-diabetic drugs that enhance cellular responsiveness to insulin. However, in some patients, fluid retention, plasma volume expansion, and edema have been observed. It is well established that insulin regulates Na⁺ reabsorption via the epithelial sodium channel (ENaC) located in the distal tubule. Therefore, we hypothesized that these agonists may positively modulate insulin-stimulated ENaC activity leading to increased Na⁺ reabsorption and fluid retention. Using electrophysiological techniques, dose-response curves for insulin-mediated Na⁺ transport in the A6, M-1, and mpkCCD_{cl4} cell lines were performed. Each line demonstrated hormone efficacy within physiological concentration ranges and, therefore, can be used to monitor clinically relevant effects of pharmacological agents which may affect electrolyte transport. Immunodetection and quantitative PCR analyses showed that each cell line expresses viable and functional PPAR γ receptors. Despite this finding, two PPAR γ agonists, pioglitazone and GW7845 did not directly enhance basal or insulin-stimulated Na⁺ flux via ENaC, as shown by electrophysiological methodologies. These studies provide important results, which eliminate insulin-mediated ENaC activation as a candidate mechanism underlying the fluid retention observed with PPAR γ agonist use.

Keywords Pioglitazone · Renal principal cell · Fluid retention · Insulin-sensitizing agents · SGK

Introduction

There is an ever-increasing need for well-defined in vitro model systems which can be used to investigate intracellular signaling mechanisms within specific cell types of organ systems. The kidney exemplifies a complex organ in which the epithelial cells lining the tubules show segment-specific differences in transepithelial resistance and permeability as well as expression of distinct hormone receptors and transport proteins.

Cultured cell models expressing the in vivo characteristics of various tubular segments have been developed and serve as valuable models for studies that would not be possible in the context of the variability present in the whole organ. In the cortical collecting duct, the principal cells are responsive to three different hormones: aldosterone, antidiuretic hormone (ADH), and insulin/IGF1 (insulin-like growth factor 1). Each of these hormones positively regulates the epithelial Na⁺ channel (ENaC), and, therefore, plays a crucial role in overall salt and water homeostasis.

While the pharmacology of the aldosterone response is relatively well defined in the available principal cell culture models, the responses to insulin are less well characterized. There is an emerging appreciation for the potential role of insulin in the development of hypertension in hyperinsulinemic states, thereby underscoring the need for well-characterized cell lines that can be used to model these clinical observations. Such cultured lines can also be utilized to investigate the efficacy and mechanism of action of pharmaceutical agents that target insulin-stimulated pathways.

Peroxisome proliferator-activated receptor gamma (PPAR γ) is a nuclear transcription factor that, when activated, plays a functional role in free fatty acid metabolism and adipocyte differentiation [35]. The

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thiazolidinedione (TZD) class of therapeutic compounds are high-affinity ligands for PPAR γ [22], which have been shown to enhance cellular responsiveness to insulin, increase insulin-dependent glucose disposal, and improve hepatic sensitivity to insulin with overall durable improvement in glucose homeostasis. There are two FDA-approved TZDs—rosiglitazone (Avandia) and pioglitazone (Actos)—used in the treatment of type 2 diabetes mellitus (T2DM) either alone or in combination with metformin, sulfonylureas or insulin.

When used as monotherapy, there are reports of TZDs causing fluid retention that can result in plasma volume expansion and, in some patients, overt edema. The incidence of edema is increased when either of these agents is used in combination with insulin [27]. However, the mechanisms involved in the fluid retentive effects have not been well described. Interestingly, while the agents cause fluid retention, they also exhibit blood pressure lowering activity in normotensive and hypertensive rodents and humans [5, 6, 21, 32].

One potential target tissue for the fluid retentive action of these ligand-activated transcription factors is the vasculature. PPAR γ is expressed in endothelial and smooth muscle cells [7, 21] and receptor activation appears to have direct vasodilatory effects in humans and rodents [5, 15, 28]. Even with changes in the vasculature, the observed clinical symptoms of salt and water retention suggest a primary or secondary effect on renal salt and water homeostasis. Localization of PPAR γ in the glomerular mesangial cells [16, 18, 20] and the distal portions of the nephron [16, 19, 36] suggest a potential for direct effects of PPAR γ agonists on renal function.

The current studies were designed to characterize the dose–response relationships for insulin-stimulated Na⁺ transport in several principal cell culture models including the A6 (derived from *Xenopus laevis*) [3] and murine M-1 [33] and mouse principal kidney cortical collecting duct (mpkCCD_{cl4}) [2] cell lines. Subsequently, the cell lines were used to examine the potential renal involvement of PPAR γ agonist-induced edema formation and to determine whether insulin's exacerbation of this effect could be manifested via Na⁺ retention.

Materials and methods

Drugs and cell lines

The A6 and M-1 cell lines were obtained from ATCC (Rockville, MD, USA). The mpkCCD_{cl4} cell line was provided by Dr. Alain Vandewalle (INSERM U478, Paris, France). Porcine insulin was obtained from Eli Lilly Corporation (Indianapolis, IN, USA). Amiloride was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pioglitazone and GW7845 were provided by GlaxoSmithKline (Research Triangle Park, NC, USA).

Cell culture

The A6 cell line was grown at 27°C in a humidified incubator gassed with 4.5% CO₂. Culture media consisted of seven parts Coon's F12 High Zinc and three parts Leibovitz's L15 (Irvine Scientific; Santa Ana, CA, USA), supplemented with 10% calf serum (ICN Biochemicals Inc., Irvine, CA, USA), 25 mM NaHCO₃, 25 U/ml penicillin, 25 mg/ml streptomycin, 1 mM Glutamax (Invitrogen; Carlsbad, CA, USA), and 12 mg/l ciprofloxacin (Voigt Global Distribution; Kansas City, MO, USA). The mammalian cell lines (M-1 and mpkCCD_{cl4}) were grown at 37°C in a humidified incubator gassed with 5% CO₂. Culture media consisted of DMEM/F12 base media (Invitrogen) supplemented with 5% fetal bovine serum (ICN Biochemicals Inc.), 1 mM Glutamax, 25 U/ml penicillin, 25 mg/ml streptomycin, and 12 mg/l ciprofloxacin. For all cell lines, media was replaced every 2 days. Cell cultures were maintained in plastic flasks until confluent and subcultured at a 1 : 10 dilution. For electrophysiological experiments, cells were subcultured onto permeable supports (Costar Transwells; Fisher, Chicago, IL, USA) at confluent density. All cells were grown on permeable supports for at least 14 days prior to electrophysiological studies.

Electrophysiology

Transwell filters containing confluent cell monolayers were clamped in a Ussing chamber and connected to a voltage clamp apparatus (WPI; Sarasota, FL, USA) via current and voltage electrodes. The initial transepithelial voltage was clamped to zero and the resulting short-circuit current (SCC) measured. The cells were bathed with the appropriate serum-free media, which was maintained at either 27 or 37°C for the A6 cell line and mammalian cell lines, respectively. A 5/95% CO₂/O₂ gas lift served to circulate the bathing media, as well as maintain pH. All cell lines were serum-starved for 18–24 h prior to electrophysiological studies. Either pioglitazone (10 μ M) or GW7845 (100 nM) was added to the serosal bathing media 18–24 h prior to and during electrophysiological studies. In all experiments, insulin was added to the serosal bathing media and amiloride (10^{−5} M) was added to the apical bathing media 30 min after insulin addition. Transepithelial resistance (an indication of cellular viability) was monitored throughout the entire duration of each electrophysiological experiment by pulsing the tissues with a 2,000 μ V pulse every 200 s. Resistance values were calculated from the resulting current deflections using Ohm's law. By convention, an increase in SCC is indicative of cation absorption (apical to basal flux) or of anion secretion (basal to apical flux). A6 and mpkCCD_{cl4} cells were only used in statistical analyses if tissue resistances were $\geq 1,000 \Omega\cdot\text{cm}^2$. M-1 cells were only included in statistical analyses if tissue resistances were $\geq 500 \Omega\cdot\text{cm}^2$.

Total RNA extraction, reverse transcription, and quantitative polymerase-chain reaction

Total RNA from untreated M-1 and mpkCCD_{cl4} cells grown on plastic (for PPAR γ gene expression analyses) or from PPAR γ -treated mpkCCD_{cl4} cells grown on semi-permeable supports (for CD36 and SGK gene expression analyses) were extracted using RNeasy mini column (Qiagen; Valencia, CA, USA) and digested with DNase (Qiagen) following the manufacturer's instructions to remove DNA contamination. One microgram of cleaned total RNA was reverse transcribed in 100 μ l reaction using the High Capacity cDNA Archive Kit (Applied Biosystems; Foster City, CA, USA). Quantitative polymerase-chain reaction (qPCR) was performed and analyzed using ABI PRISM 7000 Sequence Detection System (Applied Biosystems) with gene-specific primers and probes. Mouse-specific oligonucleotides used in qPCR analyses are outlined in Table 1.

Gel electrophoresis and western blotting

Cells grown on permeable supports were washed in ice-cold serum-free culture media and solubilized with lysis buffer (4% SDS, 10% glycerol, and 1 mM DTT in 0.05 M Tris, pH 6.8). Lysates were sonicated for 20 1-s pulses with a titanium-tip sonicator (20% duty cycle, Branson Sonifier 450). Protein concentration was determined using the DC protein assay kit (Biorad; Hercules, CA, USA). The cell lysates were separated by SDS-PAGE on 7.5% acrylamide gels and blotted onto Immobilon-P transfer membrane (Millipore Corp.; Bedford, MA, USA). The membranes were blocked with 5% milk-TBS, pH 7.5 and subsequently incubated overnight at 4°C with gentle agitation with either an anti-PPAR γ antibody (Affinity BioReagents; Golden, CO) at a 1:3,000 dilution, an anti-CD36 antibody (Cayman Chemical; Ann Arbor, MI) at a 1:1,000 dilution, or an anti-SGK antibody (Dr. Nicola Perrotti; Univ. Magna Graecia of Catanzaro, Catanzaro, Italy) at a 1:1,000 dilution followed by incubation with anti-rabbit antibody conjugated to horseradish peroxidase (Upstate Inc.; Charlottesville, VA, USA) at a 1:10,000 or 1:60,000 dilution. Both antibodies were diluted in 0.5% milk-TBS, pH 7.5. The protein bands were visualized with SuperSignal West Dura enhanced chemiluminescence reagent (Pierce; Rockford, IL, USA) and developed on film.

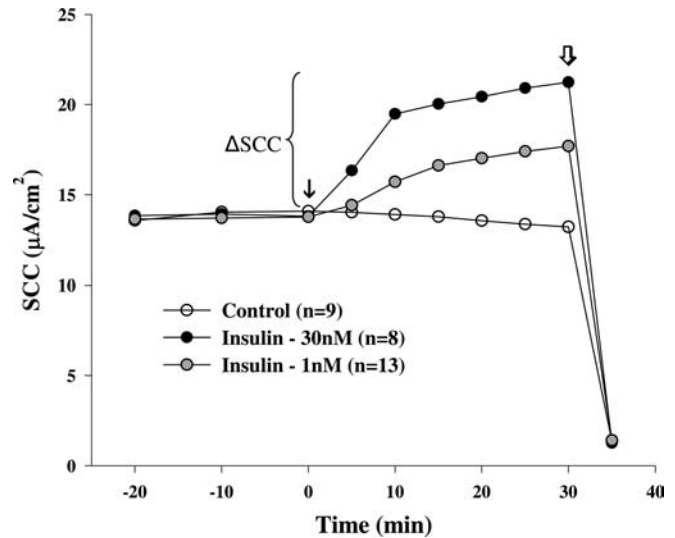


Fig. 1 Insulin-stimulated Na⁺ reabsorption in A6 cells. Confluent cultures were serum-starved 18–24 h. The *solid arrow* indicates time of insulin addition. The *open arrow* indicates time of amiloride (10⁻⁵ M) addition. Varying insulin concentrations result in concomitant rises in net SCC. The magnitude (Δ SCC) of reabsorptive Na⁺ flux stimulated by 30 nM insulin is indicated by the bracket ($SCC_{t=30 \text{ min}} - SCC_{t=0 \text{ min}}$). Symbols represent the mean of *n* experiments. The standard error bars were not included for clarity. Significance of the responses are shown in Fig. 2

Statistical analyses

Symbols and/or bar graphs are represented as means \pm standard error of the mean (SEM). Differences between groups were evaluated using Student's *t*-test. The *p* values are defined in the figure legends.

Results

In cultured cells, electrogenic transport such as trans-epithelial Na⁺ flux can be measured by clamping the spontaneous potential difference of a confluent, high-resistance monolayer to zero and recording the SCC. Ion transport generated by Na⁺ flux in the reabsorptive direction can be quantitated with the use of an ENaC-specific inhibitor, amiloride. Representative ion transport responses of the A6 cell line to maximal and sub-maximal concentrations of insulin are shown in Fig. 1. In this cell line, both basal (control) and insulin-stimulated ion flux are predominantly amiloride-sensitive. The ion transport traces of the M-1 and mpkCCD_{cl4} cells are similar to that seen in the A6 cells, except that the M-1

Table 1 Primer sets and probes: oligonucleotides used for qPCR analyses

Gene	Forward primer	Reverse primer	Probe
PPAR γ	tgaataaagatggagtcctcatctca	ggcttcgcaggttttga	aggccaaggattcatgaccagg
CD36	cctgcagaaatactagccaacacc	tgagtcctatgcagttcc	cgaaaacgctggcttctgtataccga
SGK	cgggtgactggtgtgtctt	agccgtgttccgctataaa	ctgtctgtatgagatgctctacggcctg

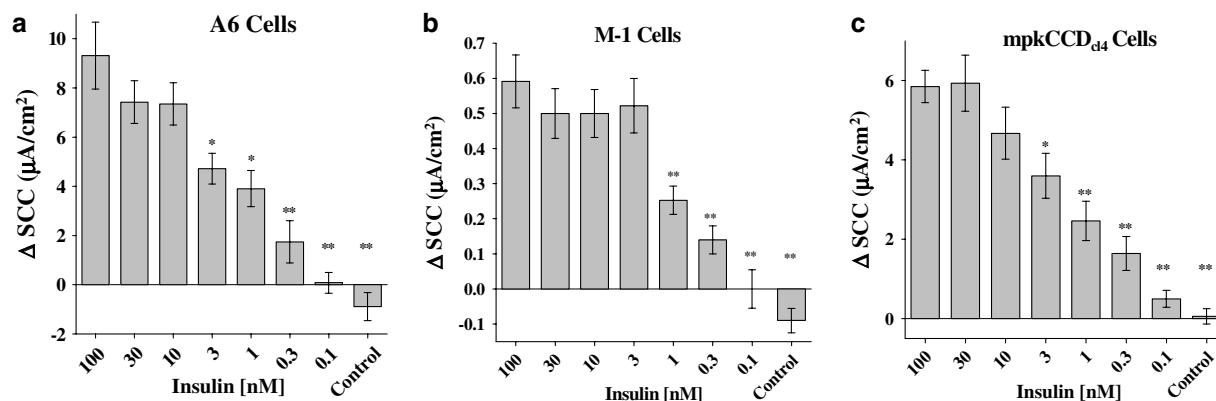


Fig. 2 Insulin dose-response curves in principal cell culture models. (a) A6, (b) M-1, and (c) mpkCCD_{cl4} cells were serum-starved 18–24 h and subsequently stimulated with varying concentrations of insulin. The magnitude of insulin-stimulated Na⁺ flux is depicted in bar graphs representing means \pm SEM and are measured as ($\Delta\text{SCC} = \text{SCC}_{t=30 \text{ min}} - \text{SCC}_{t=0 \text{ min}}$) in the case of A6

and mpkCCD_{cl4} cells, and as ($\Delta\text{SCC} = \text{SCC}_{t=10 \text{ min}} - \text{SCC}_{t=0 \text{ min}}$) for the M-1 cells. The number of experiments for each insulin dose are between 5 and 13, 5 and 14, and 5 and 11 for the A6, M-1, and mpkCCD_{cl4} cells, respectively. Statistical significance compared to the maximal insulin dose (30 nM) is denoted using Student's *t*-test; * $p \leq 0.05$, ** $p \leq 0.01$

cells display a lower rate of basal ion transport as compared to the other two cell lines (data not shown).

The dose-response relationships between Na⁺ flux and insulin concentrations for all three cell lines are expressed as the insulin-stimulated increase over baseline (ΔSCC) in Fig. 2. Under these experimental conditions, the rise in SCC elicited by 30 nM insulin is maximal in all cell lines. The dose-response curves elicited in the amphibian and mammalian cell lines are virtually identical. However, in the M-1 cells, the magnitude of the insulin-stimulated changes in net transport are approximately tenfold less than that observed in either the A6 or mpkCCD_{cl4} cells (compare the SCC scales between the panels in Fig. 2). The rate of basal transport observed in the M-1 cell line was also proportionally lower. Each cell line exhibits a dose-responsive relationship of insulin-stimulated Na⁺ transport within normal rodent plasma insulin concentrations (Table 2).

We performed qPCR on RNA isolated from confluent M-1 and mpkCCD_{cl4} cells and measured basal amounts of PPAR γ mRNA expression. Both cell lines express similar amounts of PPAR γ transcript in the basal state (Fig. 3a). Concurrently, a band corresponding to PPAR γ protein was detected in the A6, M-1, and mpkCCD_{cl4} cell lines (Fig. 3b). The M-1 cells express a higher level of PPAR γ protein as compared to the mpkCCD_{cl4} cells.

Table 2 Rat insulin concentrations

	Fasted	Nonfasted
Control	0.26 nM [34]	0.36 nM [34]
Fructose-fed (hyperinsulinemic)	0.71 nM [34]	1.41 nM [34]
Control	0.26 nM [14]	
Insulin infusion (hyperinsulinemic)	0.71 nM [14]	

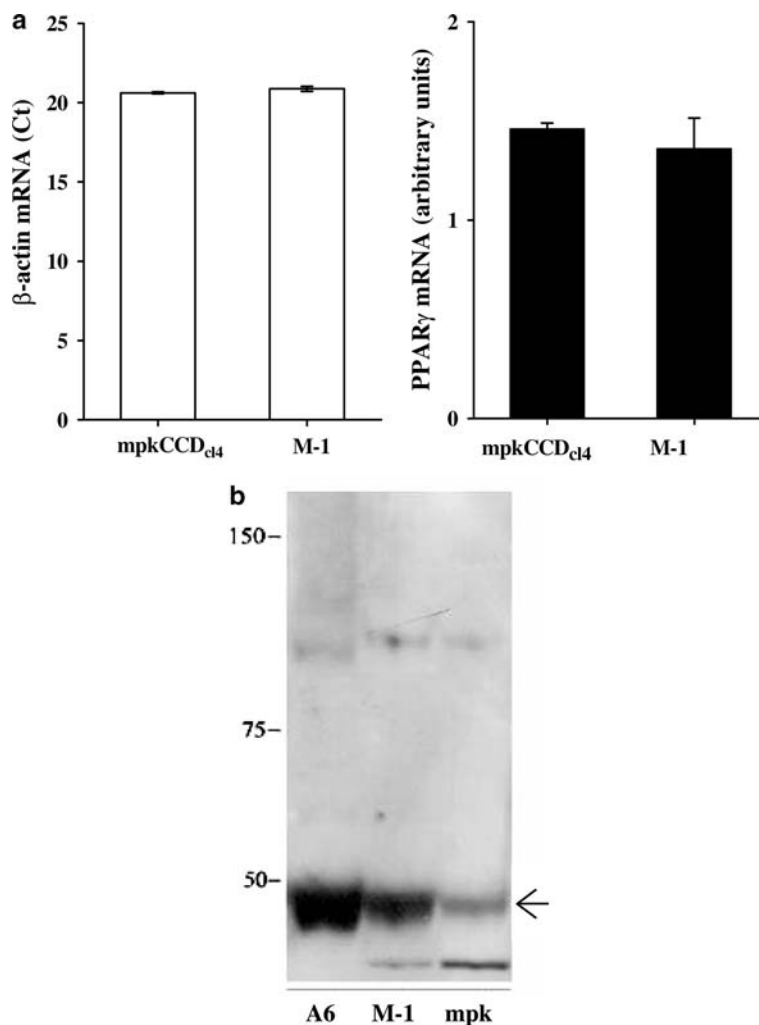
Values represent insulin concentrations in rats during control and hyperinsulinemic situations.

In order to verify that the agonists used in these studies were, in fact, functional, PPAR γ -induced gene expression was determined using the mpkCCD_{cl4} cells. CD36 is a protein commonly upregulated by PPAR γ in multiple tissues [25]. Long-term (18–24 h) incubation with either pioglitazone or GW7845 induced a significant increase in CD36 mRNA (Fig. 4a). Concurrently, CD36 protein abundance was induced upon treatment with either of the two agonists (Fig. 4b). The effect of the two agonists on expression of serum and glucocorticoid induced kinase (SGK)—an enzyme that was previously reported to be upregulated with PPAR γ agonist treatment [19]—was also investigated in the mpkCCD_{cl4} cells (Fig. 4). We did not observe any changes in the levels of SGK transcript or protein expression after a long-term (18–24 h) incubation with either PPAR γ agonist.

The majority of basal ion transport in each cell line is amiloride-sensitive, indicating that it is predominately due to reabsorptive Na⁺ movement via ENaC. It is possible that the PPAR γ agonists may affect salt and water homeostasis by modulating basal ENaC activity rather than by regulating hormonally induced changes in channel transport. However, differences in the amount of basal Na⁺ transport were not observed in either the A6 or the mpkCCD_{cl4} cell lines after long-term (18–24 h) pioglitazone or GW7845 treatment. The effect of short-term (2 h) agonist incubation was examined in the mpkCCD_{cl4} cell line. Neither agonist affected basal Na⁺ transport after 2 h (data not shown). On the other hand, long-term treatment with GW7845 significantly decreased basal ion transport compared to the untreated tissue in the M-1 cell line (Fig. 5). The explanation for the anomalous GW7845-mediated decrease in basal ion flux in the M-1 cell line and not the other two cell lines has not yet been determined.

To ascertain potential insulin-sensitizing effects of the PPAR γ agonists, a submaximal (1 nM) insulin concentration was used to stimulate cells preincubated

Fig. 3 Basal PPAR γ expression levels in M-1 and mpkCCD_{cl4} cells. **(a)** The expression of PPAR γ mRNA in M-1 and mpkCCD_{cl4} cells was normalized to the expression of β -actin mRNA and presented as arbitrary units. **(b)** PPAR γ protein expression in A6, M-1, and mpkCCD_{cl4} cells. Confluent monolayers of each cell line were lysed, separated by SDS-PAGE, blotted onto transfer membrane, and probed for PPAR γ . Equal amounts of protein (100 μ g) was loaded in each lane. The *arrow* shows an \sim 47 kDa protein corresponding to PPAR γ . Results are representative of three independent experiments



(18–24 h) in the presence or absence of either agonist. Neither pioglitazone nor GW7845 treatment significantly increased the amount of Na⁺ flux elicited by a submaximal (1 nM) insulin dose in any of the three cell lines (Fig. 6).

Discussion

Monogenetic aberrations in proteins belonging to the hormonal axes that regulate ENaC activity have been shown to cause changes in salt and fluid balance leading to hypertensive or hypotensive states [9, 31]. Insulin enhances Na⁺ reabsorption in the distal tubule and, in humans, there is a correlation between insulin resistance, hyperinsulinemia and hypertension that is independent of age, gender, or degree of obesity [8, 11, 12, 24, 29, 30]. The *in vivo* studies have been substantiated by work in cell culture models where it has been conclusively shown that insulin regulates ENaC activity and cell surface expression [1, 3, 4].

We focused our study on the potential for direct effects of PPAR γ activation in the regulation of salt and water

balance in the principal cells of the distal tubule. Interestingly, like insulin-mediated insertion of GLUT-4 transporters into the plasma membrane of adipose and skeletal muscle cells [10, 37], insulin-mediated insertion of ENaC into the apical membrane of principal cells is under the control of the phosphoinositide (PI) pathway [3, 4, 13]. Therefore, we postulated that PPAR γ ligands may have actions not only on cellular glucose uptake but also on expression and cellular localization of proteins that regulate other transport processes such as ENaC-mediated Na⁺ absorption. In addition, PPAR γ is found in the distal tubule and it is conceivable that the volume expansion observed with agonist treatment may be linked to the natriuretic effects of insulin or, alternatively, may be the result of a direct PPAR γ effect on Na⁺ transport.

The significant findings from this investigation include: (1) net ENaC-mediated Na⁺ flux in three distinct *in vitro* models of the renal principal cell type exhibit similar dose-dependent regulation by insulin; (2) the three cell lines express functional PPAR γ and are, therefore, capable of modulation by PPAR γ -activating agents; and (3) activation of PPAR γ with either pioglitazone (a true TZD) or the more potent PPAR γ ago-

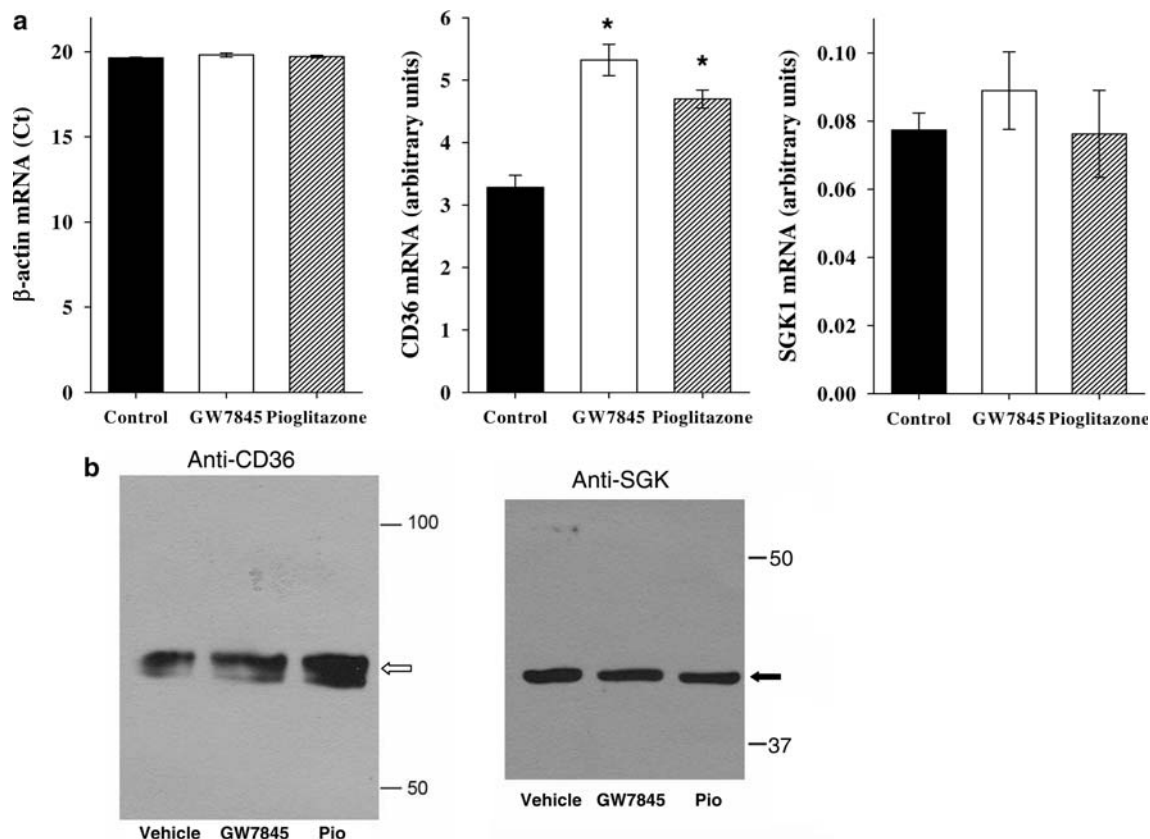


Fig. 4 Effect of PPAR γ agonists on CD36 and SGK expression. Confluent monolayers of mpkCCD_{cl4} cells were serum-starved overnight and treated with either vehicle (black bars), 100 nM GW7845 (white bars), or 10 μ M pioglitazone (hashed bars) 18–24 h. (a) CD36 mRNA or SGK mRNA was normalized to the expression of β -actin mRNA and presented as arbitrary units. Bars represent means \pm SEM. Statistical significance in gene expression between PPAR γ -treated tissues and vehicle-treated tissues for CD36 mRNA is denoted using Student's *t*-test;

* $p \leq 0.05$. Results are representative of two independent experiments. (b) Whole cell lysates of serum-starved mpkCCD_{cl4} cells treated 18–24 h with either vehicle (DMSO), pioglitazone (10 μ M), or GW7845 (100 nM) were separated by SDS-PAGE, blotted onto transfer membrane, and probed for CD36 or SGK. Equal amounts of protein (100 μ g) was loaded in each lane. The open arrow shows a doublet migrating at \sim 80 kDa corresponding to CD36, whereas the solid arrow shows a band migrating at \sim 46 kDa corresponding to SGK. Results are representative of two individual experiments

nist, GW7845 (a non-TZD), did not change the magnitude of (a) basal Na⁺ transport in two of the cell lines (A6 and mpkCCD_{cl4}) or (b) insulin-stimulated Na⁺ transport in any of the cell lines.

There is a striking similarity between the cell lines with respect to the insulin dose–response curves for activation of Na⁺ transport. These studies represent the first demonstration of insulin dose–response relationships for the murine cell lines (M-1 and mpkCCD_{cl4}). The insulin concentration eliciting an approximately half-maximal response (0.3 nM) in each cell line is within normal insulin levels for both the fasted and non-fasted states in rats (compare Table 2 with Fig. 2), demonstrating that the insulin dose–response relationship in these murine cell lines shows efficacy in the physiological range. The same is true for the A6 cell line.

The discrepancy between the net magnitude of insulin-stimulated Na⁺ flux in the A6, M-1, and mpkCCD_{cl4} cells remains elusive; but it should be noted that these differences are not unique to insulin. The magnitude of the natriferic responses elicited by aldosterone and ADH vary considerably from one cell line to another but, in

general, follow the same trend observed in the insulin studies. The explanation may lie in the amount and/or activity of ENaCs, or, alternatively, may be dictated by the amount of ENaC regulatory components that each line expresses.

Previous studies utilizing tissue from dissected nephrons have found PPAR γ expression in various segments of the distal tubule. In situ hybridization has shown the expression of PPAR γ transcripts in human, rabbit, and rat collecting duct (CD) [17, 19, 36]. Immunohistochemical studies have indicated the presence of the PPAR γ protein in freshly isolated rabbit inner medullary collecting duct (IMDC) and in primary cultures of IMCD as well as CCDs [17]. Recently, Hong et al. used immunohistochemical staining to show the expression of PPAR γ protein in the distal convoluted tubule, CCDs, and medullary CDs in human kidney sections. In addition, this group detected PPAR γ transcripts in a mouse IMCD cell line (mIMCD-K2) as well as in M-1 and human CCD principal cell lines [19].

We detected transcripts for PPAR γ in the mpkCCD_{cl4} and M-1 cells. The A6 cell line was excluded

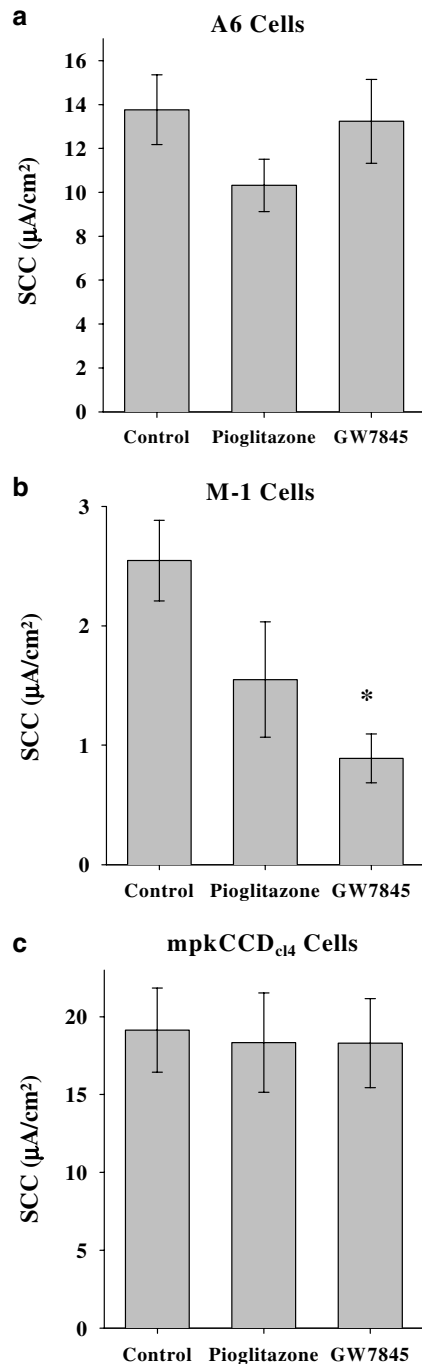


Fig. 5 Effect of pioglitazone and GW7845 on basal ion transport. (a) A6 cells, (b) M-1 cells, and (c) mpkCCD_{cl4} cells were serum-starved overnight and treated with either pioglitazone (10 μM) or GW7845 (100 nM) 18–24 h. Basal transport rates just before addition of insulin (time = 0) in control, pioglitazone, or GW7845 treated cells were compared. Bars depict means \pm SEM. The number of experiments for each condition (control or PPAR γ -treated) range between 8 and 13, 4 and 11, and 9 and 11 for A6, M-1, and mpkCCD_{cl4} cells, respectively. Statistical significance compared to the untreated cells is denoted using Student's *t*-test; **p* \leq 0.05

from this analysis due to lack of species-specific primers. The results in the M-1 cell line substantiate the previous findings of Yang et al., while the results in the

mpkCCD_{cl4} cell line are novel. Each cell line also expresses PPAR γ protein in varying amounts. Interestingly, the PPAR γ protein expression in the M-1 and mpkCCD_{cl4} cells does not follow the same trend as the mRNA expression. This observation may explain the baseline-lowering effect of GW7845 in the M-1, but not mpkCCD_{cl4} cell line (Fig. 3b, c). However, this reasoning is not consistent when considering the protein expression of PPAR γ in the A6 cells. GW7845 did not cause a decline in basal transport in this line (Fig. 3a) even though the A6 cells express the highest amount of PPAR γ protein.

We next explored the functionality of the PPAR γ agonists used in these studies. qPCR was used to assess PPAR γ -induced gene expression in the mpkCCD_{cl4} cells. This line was chosen for the gene analyses due to the availability of species-specific primer sets, as well as the robust nature of their natriuretic responses to insulin. CD36 is a transmembrane glycoprotein involved in foam cell formation and this protein has been shown to be upregulated in response to PPAR γ treatment [25]. Pioglitazone and GW7845 both caused a significant increase in CD36 mRNA and protein expression, verifying the functionality of the PPAR γ agonists.

In summary, PPAR γ is present in the sections of the kidney containing principal cells as well as in cultured principal cell lines. It has been demonstrated that each cell line utilized in this study also expresses the protein and/or mRNA for each of the ENaC subunits [2, 23, 26]. Thus, the key components of the potential PPAR γ -regulated pathway are expressed in the cell lines used.

Our studies suggest that the TZDs are not contributing to the fluid retention through direct regulation of ENaC or insulin-stimulated ENaC activity. It is possible that the agonists may be acting on water and Na⁺ transporting machinery located prior to the distal convoluted tubule. Rosiglitazone treatment in Sprague-Dawley rats was shown to increase protein abundance of the sodium-hydrogen exchanger-3 (NHE3), the primary source of Na⁺ reabsorption in the proximal tubule [32]. However, in the same study, expression of the collecting duct aquaporins 2 and 3 were shown to be up-regulated when challenged with rosiglitazone, suggesting that ADH may have a role in PPAR γ -mediated fluid retention and, in agreement with the previously cited papers, indicate a distal site of action. Also consistent with a distal effect are the observations of Hong and colleagues, who showed that treatment of a PPAR γ -expressing human CCD cell line with either rosiglitazone (2 μM) or pioglitazone (20 μM) increased the translocation of αENaC to the plasma membrane, although the functional ramifications of this observation were not explored [19].

In the Hong et al. study, the translocation of αENaC was accompanied by an upregulation of SGK mRNA and activity within 4 h of PPAR γ agonist treatment. SGK is a downstream enzyme activated in the PI pathway—a signaling system shared by various

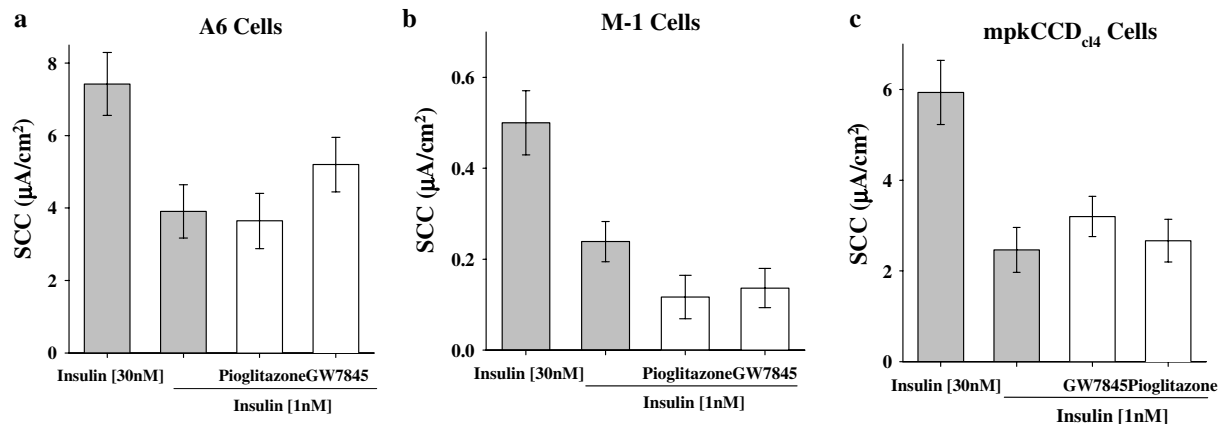


Fig. 6 Effect of PPAR γ agonists on Na⁺ transport stimulated by a submaximal (1 nM) insulin dose. Either pioglitazone (10 μ M) or GW7845 (100 nM) was added to the serosal bathing media of confluent (a) A6, (b) M-1, or (c) mpkCCD_{cl4} cells 18–24 h prior to and during the electrophysiological studies. The magnitude of Na⁺ flux stimulated by a submaximal (1 nM) insulin dose is depicted in bar graphs representing means \pm SEM and are measured as (Δ SCC = SCC_{t=30 min} – SCC_{t=0 min}) in the case of A6 and

mpkCCD_{cl4} cells, and as (Δ SCC = SCC_{t=10 min} – SCC_{t=0 min}) for M-1 cells. The number of experiments for each experimental condition (insulin alone or insulin plus PPAR γ agonist) range between 8 and 13, 5 and 14, and 9 and 11 for A6, M-1, and mpkCCD_{cl4} cells, respectively. Statistical analyses indicated that neither pioglitazone nor GW7845 had a significant effect on the 1 nM insulin response in any of the cell lines

ENaC-regulating hormones. In functional studies, over-expression of wild-type SGK has been shown to increase basal, but not insulin-stimulated Na⁺ transport through ENaC [13]. We did not observe PPAR γ -induced changes in the amount of SGK transcript or expressed protein in the mpkCCD_{cl4} cells. In addition, neither PPAR γ agonist induced basal or insulin-stimulated ENaC activity in the same cell line. It is possible that the PPAR γ agonists may regulate SGK activity by a post-translational mechanism. If this is the case, one would expect PPAR γ agonist treatment to positively regulate basal ENaC activity; however in the mpkCCD_{cl4} cells, we did not find significant increases in basal ENaC transport either 2 h (data not shown) or 18–24 h (Fig. 5) after treatment with GW7845 (100 nM) or pioglitazone (10 μ M). The differences in the experimental results between the Hong et al. study and the present study may be explained by differences in the cell lines utilized and/or PPAR γ agonist concentrations tested.

On the other hand, SGK has been implicated as both necessary and rate-limiting for ADH-stimulated Na⁺ transport [13]. Therefore, it is entirely possible that the PPAR γ agonists may be modulating ENaC and/or other transporter function via other hormonal pathways independent of insulin.

We have verified that three cell culture models of the principal cell type respond to physiologically relevant insulin concentrations in a dose-dependent manner. In addition, we have shown that PPAR γ agonists do not potentiate insulin-mediated Na⁺ flux in any of the lines, suggesting that PPAR γ activation does not regulate fluid homeostasis via a direct effect on insulin-stimulated ENaC activity in isolated cell lines. The *in vivo* situation may be more complex, encompassing factors that are not found in cultured cells. These findings are important

in ruling out targets for clinical intervention of the fluid retention and will provide the basis for future studies involving the regulation of other renal transporter functions by PPAR γ .

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